College of Arts and Sciences / Department of Molecular Genetics

Promoter Mutation in *Ccna2* Reveals Novel Functions of the Protein in Spermatogenesis

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INTRODUCTION

The cyclins are a set of proteins that play a key role in regulating the cell cycle by activating the CDKs, which in part coordinate the events that lead to cellular division (both mitotic and meiotic). Cyclin A2 (CCNA2) is the regulatory subunit of CDK1 and 2; together, they phosphorylate specific proteins at the S phase and during the G2/M transition.[1]

During spermatogenesis (Fig 3C), germ cells called Spermatogonial Stem Cells (SSCs) undergo subsequent differentiation events until spermatozoa are produced. SSCs are the main cell type that expresses *Ccna2*, and are the source of continuous production of sperm.[2] Interestingly, during differentiation, *Ccna2* expression is downregulated in order for subsequent meiotic events to occur.[3]

This temporal specificity of *Ccna2* expression in the cell cycle is mediated mostly by the E2Fs, a family of transcription factors that regulate the expression of many cell cycle related genes.[4] The relevance of the precise timing and expression level of E2F-driven *Ccna2* expression has not been thoroughly studied *in vivo.*

To evaluate the roll of E2F-mediated regulation of *Ccna2 in vivo*, we have generated mice with a mutated E2F binding site in the promoter of *Ccna2 (Ccna2^{m})*. Although mice are viable and appear healthy, we observed infertility and testicular atrophy in males homozygous for the promoter mutation (Fig 2B).

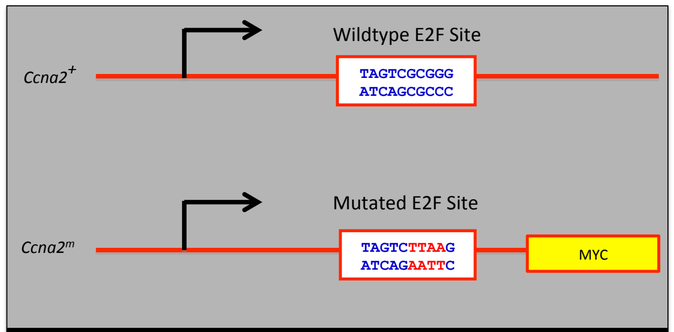

Fig 1: Diagram of mouse lines. The wildtype locus of *Ccna2* (*Ccna2^{+}*) has an E2F site 11 base pairs from the transcription start site. The mouse line generated (*Ccna2^{m}*) has four base pairs mutated at the binding site and a 5xMYC tag inserted after the translation start site.

METHODS

- A mouse line with a null E2F binding site at the *Ccna2* locus was generated (Fig 1)
- Mouse viability and fertility was tracked throughout life.
- Mice were collected at 2,3,4,6 and 12 weeks of age. Testis were weighed and then fixed.
- Testicular atrophy was assessed via histological analysis and comparing testes:body weight ratios between cohorts.
- Samples were fixed with either bouin's fixative for histological analysis and formalin for marker analysis via immunohistochemistry (IHC)

A

Genotype	Fertile males (%)	Testes:body weight ratio (%)
Ccna2$^{+/+}$	100% (n=5)	0.62 % (n=5)
Ccna2$^{+/m}$	100% (n=8)	0.58 % (n=7)
Ccna2$^{m/m}$	0% (n=4)	0.19% (n=4)

B

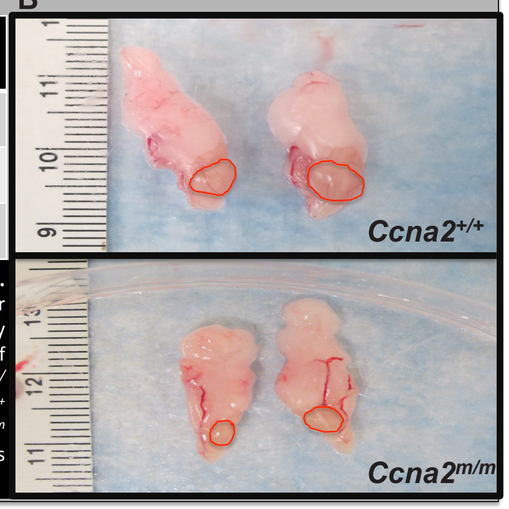

Fig 2: Adult *Ccna2$^{m/m}$* male mice are infertile and have testicular atrophy. (A) Adult male mice of each group were placed with females and their fertility was evaluated. *Ccna2$^{m/m}$* males mated successfully as measured by the presence of a copulation plug, but no litters were observed. Analysis of the male reproduction tract revealed extreme testicular atrophy in *Ccna2$^{m/m}$* adult mice as measured by the body : testes weight ratio. Both *Ccna2$^{+/+}$* and *Ccna2$^{+/m}$* mice presented normal testicular development, but *Ccna2$^{m/m}$* had a three-fold reduction of testicular mass. (B) Representative examples of 6 week-old *Ccna2$^{+/+}$* and *Ccna2$^{m/m}$* testes outlined in red.

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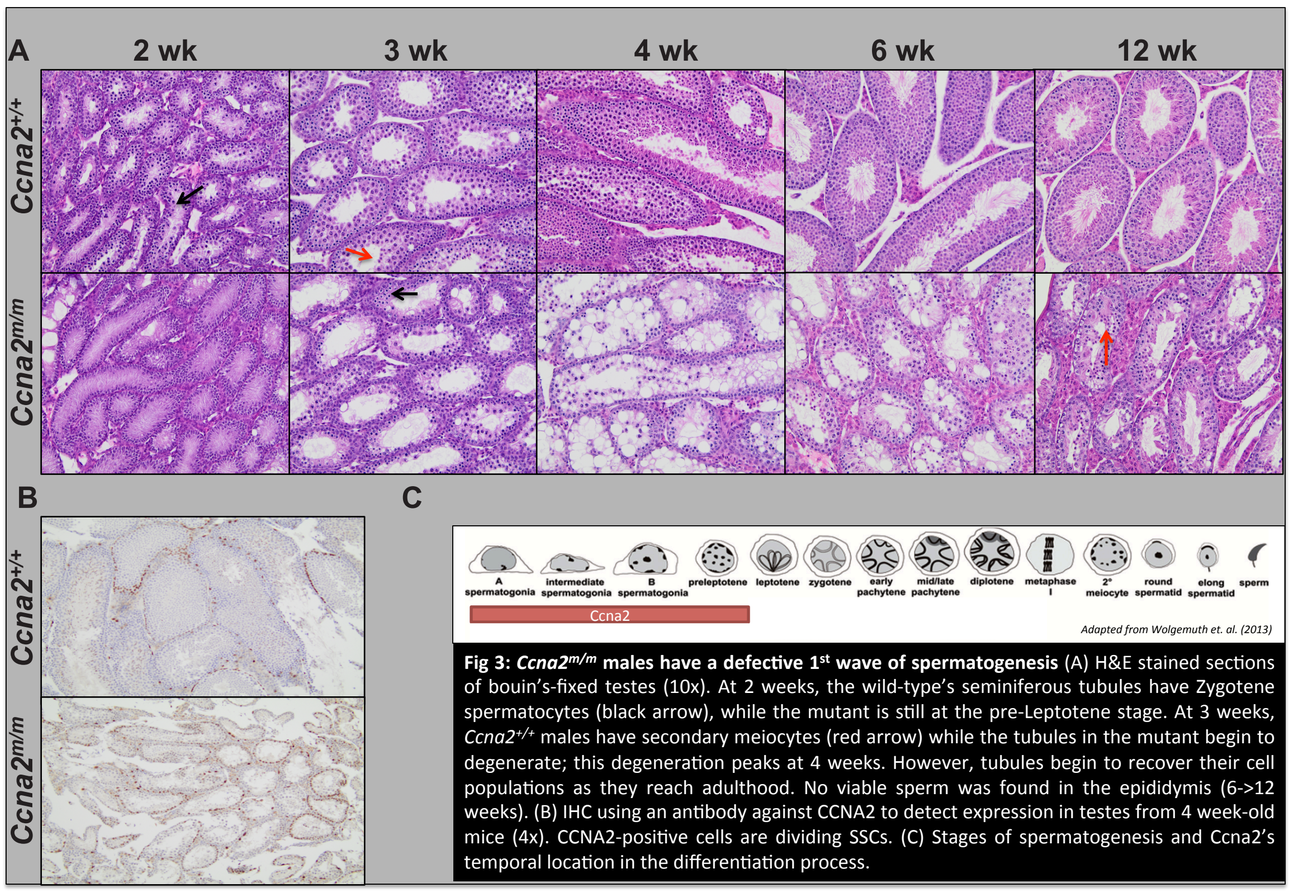

Fig 3: *Ccna2$^{m/m}$* males have a defective 1st wave of spermatogenesis (A) H&E stained sections of bouin's-fixed testes (10x). At 2 weeks, the wild-type's seminiferous tubules have Zygotene spermatocytes (black arrow), while the mutant is still at the pre-Leptotene stage. At 3 weeks, *Ccna2$^{+/+}$* males have secondary meiocytes (red arrow) while the tubules in the mutant begin to degenerate; this degeneration peaks at 4 weeks. However, tubules begin to recover their cell populations as they reach adulthood. No viable sperm was found in the epididymis (6->12 weeks). (B) IHC using an antibody against CCNA2 to detect expression in testes from 4 week-old mice (4x). CCNA2-positive cells are dividing SSCs. (C) Stages of spermatogenesis and Ccna2's temporal location in the differentiation process.

RESULTS

Analysis of breeding revealed that mutants are completely infertile, and that one copy of the functional promoter site is sufficient for fertility (Fig 2A). Histological analysis revealed a delayed 1st wave of spermatogenesis at 2 weeks followed by progressive degeneration of the seminiferous tubules; degeneration peaks at 4 weeks. Surprisingly, the seminiferous tubules begin to recover their cell populations as they reach adulthood (Fig 3A). IHC confirmed the presence of CCNA2 in mutant SSC, which indicates that the promoter mutation does not prevent CCNA2 expression (Fig 3B).

CONCLUSIONS/FUTURE PROJECTIONS

The data suggests that E2F regulation of *Ccna2* is essential for a successful 1st wave of spermatogenesis. Since cell populations seem to recover, further breeding studies will be conducted on older mice to identify if eventually there is a complete recovery of fertility (up to 24 weeks of age). The molecular pathway causing this phenotype is currently being studied through spermatid squash preparations and marker analysis via quantitative PCR (qPCR) and IHC. Preliminary data suggests that the phenotype resembles those of *Cdk2* mutant mice, but further tests are required in order to confirm this.

Bibliographies

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